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Adaptation of a Mallard H5N2 Low Pathogenicity Influenza Virus in Chickens with Prior History of Infection with Infectious Bursal Disease Virus

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SUMMARY. The influenza A/Mallard/Pennsylvania/10218/1984 (H5N2) virus is unable to replicate in 3-wk-old immunocompetent specific-pathogen-free chickens when a dose of 5×10^6 50% egg infectious dose/ml is used. In contrast, this mallard virus shows limited replication in 3-wk-old chickens that had been previously infected at 2 days of age with, and recovered from, the immunosuppressive agent infectious bursal disease virus (IBDV; herein referred to as IBDV chickens). This limited replication in IBDV chickens allowed for the serial passage of the mallard influenza virus in chickens. After 22 passages (P22) in IBDV chickens, the resulting chicken-adapted influenza virus replicated in both immunocompetent and IBDV chickens more efficiently than the mallard influenza virus. Analysis of the outcomes of infection and the lesions caused by the two viruses at the microscopic level in a time-point study showed that the P22 virus is more virulent than the parental mallard virus in both immunocompetent and IBDV chickens. Our studies provide evidence that a previous history of IBDV infection in chickens may render them more susceptible to avian influenza virus (AIV) infections, allowing for the potential introduction of AIVs in an otherwise resistant population.

RESUMEN. Adaptación de un virus de la influenza aviar de baja patogenicidad H5N2 de patos de collar en pollos con historia previa de infección por el virus de la enfermedad infecciosa de la bolsa.

El virus de la influenza A/Pato de collar/Pensilvania/10218/1984 (H5N2) es incapaz de replicarse en pollos libres de patógenos específicos de tres semanas de edad que son inmunocompetentes cuando se utiliza una dosis de 5×10^6 dosis infectantes para embrión de pollo 50% por ml. En cambio, este virus de pato de collar muestra una replicación limitada en pollos de tres semanas de edad que habían sido previamente infectados a los 2 días de edad con el virus de la enfermedad infecciosa de la bolsa y que se recuperaron de la infección por este virus inmunodepresor (en lo sucesivo denominados pollos IBDV). Esta replicación limitada en los pollos IBDV permitió el paso seriado del virus de la influenza del pato de collar en pollos. Después de 22 pasajes en los pollos IBDV, el virus de influenza resultante y adaptado a pollo se replicó en pollos inmunocompetentes y en los pollos IBDV de manera más eficiente que el virus de la influenza aviar de los patos de collar. El análisis de los resultados de la infección y de las lesiones causadas por los dos virus a nivel microscópico en un estudio en diferentes tiempos mostró que el virus con 22 pasajes es más virulento en pollos inmunocompetentes y en pollos IBDV que su progenitor el virus del pato de collar. Estos estudios aportan evidencia de que una infección previa por el virus de la enfermedad infecciosa de la bolsa en los pollos puede hacerlos más susceptibles a la infección por el virus de la influenza aviar, lo que permite la introducción potencial de este virus en una población que de otra forma sería resistente.

Key words: avian influenza, adaptation, infectious bursal disease virus, pathogenesis

Abbreviations: AI = avian influenza; AIV = avian influenza virus; AWA = Animal Welfare Act; BHI = brain-heart infusion; BLD = below level of detection; CID_{50} = 50% chicken infectious dose; dpi = days postinfection; EID_{50} = 50% egg infectious dose; ELISA = enzyme-linked immunosorbent assay; HA = hemagglutination; H&E = hematoxylin and eosin; HI = hemagglutination inhibition; HP = highly pathogenic; HPAIV = highly pathogenic avian influenza virus; IBDV = infectious bursal disease virus; LP = low pathogenic; LPAIV = low pathogenic avian influenza virus; MP = mildly pathogenic; NA = neuraminidase; Oc = ocular; P = passage; PBS = phosphate-buffered saline; SPF = specific-pathogen-free; TPB = tryptose phosphate broth; WHO = World Health Organization; WT = wild-type

Influenza type A viruses are members of the *Orthomyxoviridae* family and are divided into subtypes based on the antigenic properties of the two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) (29). To date, 16 HA subtypes and 9 NA subtypes have been characterized in influenza strains in many different combinations. It is commonly accepted that the primordial reservoir of type A influenza viruses is the wild aquatic birds of the world (29). From this large reservoir, occasionally new viruses emerge that infect other avian and mammalian species (27). The factors that determine interspecies transmission and virulence of influenza viruses in both avian and mammalian species are generally poorly understood (1). In poultry, most influenza virus infections are either subclinical or produce mild to moderate signs of disease. These avian influenza viruses (AIVs) have been categorized as low pathogenic (LP) or mildly pathogenic (MP). AIVs of the H5 and H7 subtypes can occasionally become highly pathogenic (HP) (1) causing severe systemic disease resulting in high rates of morbidity and mortality (1,3,4,7). It is commonly accepted that HPAIV strains are derived from LPAIV strains that acquire polybasic amino acid mutations within the cleavage site of the HA viral surface protein. Maturation of the influenza virion requires cleavage of the HA by

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cellular proteases, mostly trypsin-like proteases found in the lumen of the respiratory and intestinal tract of birds. The HA of HPAIV strains are instead recognized and cleaved by intracellular proteases of the subtilisin-like family associated to the Golgi apparatus, allowing the virus to cause systemic spread (2,6,17). In nature a LPAIV strain of H5 or H7 is likely introduced into susceptible poultry species from the wild aquatic avian reservoir and through several cycles of infection; these strains may undergo a series of mutational events resulting not only in adaptation to their new hosts but also into HP forms (2,16,17). Numerous laboratory studies have shown that domestic poultry species are rather resistant to infection with AIVs from aquatic birds, resulting in either poor or no replication and very limited, if any, transmission. Thus, the occurrence of AIV strains with expanded host range is perhaps limited. Ecologic, environmental, and host conditions are factors that modulate host restriction barriers for AIVs. In this regard, the host immune status is likely to modulate the susceptibility to AIV infection because it is well known that immunodeficiency leads to increases in the frequency of viral and bacterial infections (10,20). In chickens, infectious bursal disease virus (IBDV) is a common viral infection that causes permanent immunologic damage. Because IBDV targets the bursa of Fabricius, infections usually result in deficiency of B cells, the extent of which depends on the virulence of each IBDV strain. IBD is a major economic concern for the poultry industry because of its prevalence in poultry-producing areas of the world (18). Most commercial chickens are infected with IBDV early in life. Both broiler and layer flocks are susceptible to the immunosuppressive effects of the virus (18), with not only humoral but also cellular immune responses being compromised (19). Thus IBDV is an effective immunosuppressive agent and this effect is greater when chicks are infected within the first week of life (19). Interestingly, little is known about the potential effects of previous exposure to IBDV and the subsequent susceptibility to AIV infection and/or associated clinical signs, lesions, and virus shedding. The major objective of this study was to determine whether prior exposure to IBDV early in life renders chickens more susceptible to an H5N2 AIV from the wild reservoir (mallard) and whether the virus is able to adapt and increase either its tissue tropism and/or virulence in an immunocompromised host. Our studies demonstrate that chickens that have been previously infected with IBDV are more susceptible to the H5N2 AIV from mallard. More importantly, after adaptation the virus becomes more virulent in both immunosuppressed and immunocompetent chickens highlighting the potential of prior IBDV infections as a facilitator for entry of AIV in chickens.

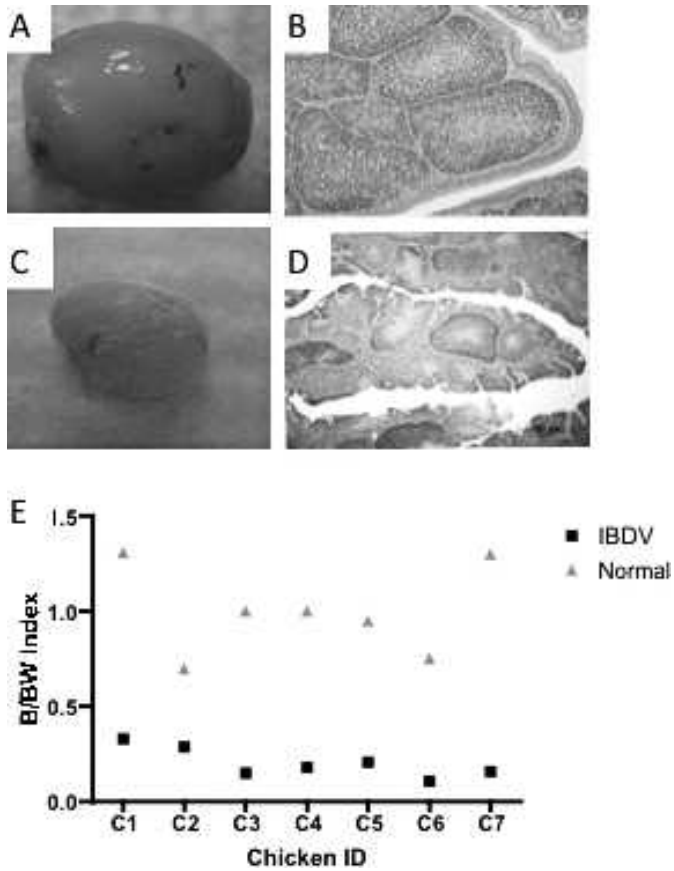


Fig. 1. Effect of IBDV infection on the bursa of Fabricius in chickens. (A) and (B) correspond to normal appearance of the bursa at the gross and microscopic (40× magnification) levels, respectively. (C) The bursa appears reduced in size and (D) shows an evident lymphoid depletion at the microscopic level in IBDV chickens (40× magnification). (E) Bursa/body weight ratio (B/BW) in immunocompetent and IBDV chickens. A value below 0.7 indicates atrophy of the bursa.

MATERIALS AND METHODS

Viruses. The A/Mallard/Pennsylvania/10218/1984 (H5N2) LPAIV (herein referred to as mal/H5N2 or wild-type [WT] AIV) used in this study was obtained from the repository at St. Jude Children's Research Hospital (Memphis, TN) as a second-passage virus in 10-day-old embryonated chicken eggs. The virus was propagated in 10-day-old embryonated specific-pathogen-free (SPF; B & E Eggs, York Springs, PA) chicken eggs and titrated to determine the 50% egg infectious dose (EID_{50}) as previously described (25). IBD-E virus stock was prepared in 3-wk-old SPF white leghorn chickens (Charles River Laboratories, Wilmington, MA) that were infected with 200 µl, equally distributed between the ocular and oral routes of IBDV stock diluted 1:10 in tryptose phosphate broth (TPB; Sigma-Aldrich, St. Louis, MO) containing an antibiotic-antimycotic solution (100×; Sigma-Aldrich). Bursa and body weights were recorded to establish the bursa/body weight index and determine the degree of virus-induced bursa atrophy (Fig. 1). Bursas were collected at 48 hr postinfection, homogenized, and tested by the IBDV antigen-capture enzyme-linked immunosorbent assay (ELISA; IBDV AC-ELISA test kit, Synbiotics Corporation, San Diego, CA) according to manufacturer's instructions. To determine the 50% chicken infectious dose (CID_{50}) for the IBDV stock, groups of three 3-wk-old SPF white leghorn chickens were inoculated with 200 µl of serial 10-fold dilutions of IBDV E-Delaware stock. Forty-eight hours postinfection, the presence of the virus in the bursa homogenate was established using the IBDV AC-ELISA assay as indicated (data not shown).

Animal studies. *H5N2 LPAIV replication in SPF white leghorn chickens.* SPF chickens were purchased from Charles River Laboratories (Wilmington, MA). Mal/H5N2 AIV was administered to groups of three 3-wk-old SPF immunocompetent chickens in doses of 1 ml at a concentration of 5×10^6 EID_{50}/ml or 1×10^8 EID_{50}/ml as indicated. Animal feeding needles (Fisher Scientific, Waltham, MA) were used to administer the virus; two drops of 50 µl each were used on each eye, 200 µl were administered intranasally, 200 µl of the virus inoculum was administered intratracheally, 200 µl orally, and 200 µl intracloacally. Tracheal and cloacal swabs were collected on alternate days from 3 to 11 days postinfection (dpi) and stored at −70 C in glass vials containing 1 ml buffered glycerol medium (50% sterile glycerol, 50% phosphate-buffered saline [PBS], 1 ml/200 ml total volume gentamicin, 10 ml 100× antibiotic-antimycotic). Swab samples were tested for the presence of virus by inoculating the swab medium in 10-day-old embryonated chicken eggs. Three eggs were used for each swab collected and 200 µl/egg were used following World Health Organization (WHO) (25) recommendations to determine the presence of virus.

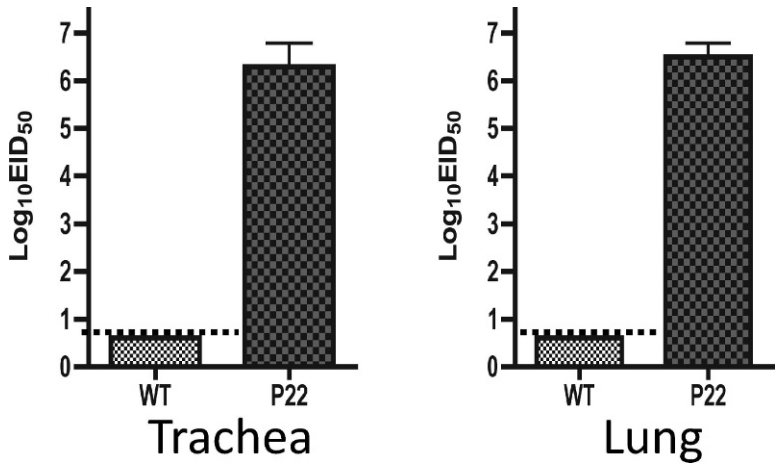


Fig. 2. Comparison of H5N2 virus replication in immunocompetent chicken. Groups of 3-wk-old SPF white leghorn chickens were infected with 5×10^6 EID_{50}/ml of either WT or P22 H5N2 AIV. Tracheal swabs and lung tissue samples were collected at 3 dpi and tested by inoculation in 10-day-old embryo chicken eggs to determine EID_{50}.

Infected eggs were incubated for 48 hr and then chilled at 4 C for no more than 24 hr or at −20 C for 30 min. Allantoic fluid was collected and a hemagglutination (HA) assay was performed as described (25). Samples showing agglutination of chicken red blood cells were scored as positive. The same approach was followed in groups of 3-wk-old IBDV chickens.

H5N2 LPAIV passages in IBDV chickens. Two-day-old SPF immunocompetent chickens were infected with 100 CID_{50} of the E-Delaware IBDV strain by oral and ocular routes. Three weeks after IBDV exposure the chickens were inoculated with the mal/H5N2 AIV. The dose for the first passage was a 1:10 dilution of mal/H5N2 viral stock that had been grown once in embryonated chicken eggs. Three IBDV chickens were used per passage. Lungs were collected at 3 dpi and a 10% (w/v) lung homogenate was prepared by adding the corresponding amount of BBL brain-heart infusion (BHI) medium (Sparks, MD). Subsequent passages were carried out by inoculation with 1 ml of pooled lung homogenates from the three chickens from the previous passage. Infection dose and routes with lung homogenates follow the same scheme presented above for H5N2 LPAIV infection. Tracheal and cloacal swabs were collected at 1 and 3 dpi and virus was detected by hemagglutination assay. Pooled lung homogenates were titrated in embryonated chicken eggs to determine virus concentration in each of the doses. The adaptation scheme consisted of 22 passages of virus in lung homogenates using 3-wk-old IBDV chickens (Fig. 2).

Pathogenesis study of the mal/H5N2 and the P22 chicken-adapted AIV. Four groups of nine 3-wk-old chickens were used in order to determine histologic changes related to differences in virulence after infection with the mal/H5N2 and the P22 chicken-adapted AIV (P22 AIV). Chickens in these groups (two groups of immunocompetent and two groups of IBDV chickens) were inoculated with 10^8 EID_{50} of either WT virus or P22 AIV. A dose of 1 ml of virus dilution was distributed via the ocular, nasal, intratracheal, and intracloacal routes. Two additional groups were mock-infected immunocompetent and IBDV chickens, and contained three birds per group. For histologic analysis, samples from a comprehensive group of organs including lungs, trachea, nasal sinus, kidney, pancreas, liver, heart, thymus, conjunctiva, bursa, and brain were systematically collected from three birds per infected group and one bird per control group at 3, 5, and 7 dpi. The animals were euthanized, necropsied, and examined for gross lesions. Tracheal and cloacal swabs were also collected in 1 ml buffered glycerol medium and stored at −70 C until use. Animal experiments were carried out under Biosafety Level 2+ conditions, with investigators wearing appropriate personal protective equipment, and were compliant with animal protocols approved by the Institutional Animal Care and Use Committee of the University of Maryland, College Park, MD, and Animal Welfare Act regulations. Birds were observed and scored daily for clinical signs of disease and general wellbeing.

Histologic examination. A time-point evaluation of histologic changes was conducted in tissues collected from groups of three chickens sacrificed at 3, 5, and 7 dpi. Tissues, including lung, trachea, nasal sinus, conjunctiva, kidney, pancreas, spleen, liver, heart, thymus, intestine, brain, and bursa of Fabricius were collected and fixed in 10% buffered formalin followed by embedding in paraffin. Sections of 5 μm were prepared and stained with hematoxylin and eosin (H&E) for microscopic examination. Scoring of lesions is as follows: +++, severe; ++, moderate; +, mild; +/−, minimal; −, no lesions.

Collection and preparation of tissue homogenates. *Bursa homogenates*. The bursa of Fabricius was extracted, washed in PBS containing an antibiotic-antimycotic solution, and weighed. A section of one-third of each sample was preserved in 10% buffered formalin for H&E staining and histologic analysis. The remaining tissue was homogenized. Briefly, an equal volume of antigen dilution buffer or TPB containing an antibiotic-antimycotic was added and a layer of sterile laboratory sea sand (Fisher Scientific) was included in the mixture to help with the grinding and homogenization. The homogenized tissue was frozen at −70 C and thawed three times, briefly mixed by vortex, and centrifuged at 1500 × *g* at 4 C for 10 min. The supernatant was collected, aliquoted into 1.5-ml microfuge tubes and stored at −70 C until use.

Lung homogenates. Lungs were extracted and washed twice in PBS-antibiotic-antimycotic solution to remove large particulate material and blood. A layer of sterile laboratory sea sand was added to the tissue and the sample was grinded using a pestle. A 10% (w/v) lung homogenate suspension was prepared by adding the corresponding amount of BHI medium containing gentamicin and antibiotic-antimycotic solution. The homogenate was centrifuged at 1500 × *g* at 4 C for 10 min and the supernatant was collected and filtered using a 0.22-μm filter unit. Aliquots of the homogenate were stored at −70 C until use.

Soft tissue homogenates: brain, kidney, intestine, pancreas, liver, and spleen. After collection the tissue was washed twice in PBS plus antibiotic-antimycotic solution and then placed in sterile tissue bags and weighed. A tissue homogenate was prepared using a laboratory blender (Seward Stomacher80, Lab System, Bohemia, NY). The samples were transported on ice and the machine was run for 1–2 min at a time until the tissue was completely homogenized. BHI medium was added to make a 10% (w/v) homogenate. The tissue homogenate was centrifuged at 1500 × *g* at 4 C for 10 min and the supernatant was filtered using a 0.22-μm filter unit. Aliquots of the homogenates were stored at −70 C until use.

Serology and hemagglutination inhibition (HI) assays. Serum samples collected from chickens after P22 H5N2 infection were tested for the presence of H5 AI and IBDV antibodies by ELISA. Commercially available, U.S. Department of Agriculture–licensed antibody test kits for the detection of antibodies to AIV (AIV antibody

Table 1. IBDV serum antibody titers determined by ELISA in 3-wk-old chickens. The ProFLOK®PLUS IBDV ELISA was used (Synbiotics).[A]

	Immunocompetent		IBDV chickens	
Chicken ID	WT (Group 1)	P22 (Group 2)	WT (Group 3)	P22 (Group 4)
1	0	0	7972	7536
2	0	0	7488	8946
3	0	0	7905	11,301
4	0	0	8436	7389
5	0	0	7409	9100
6	0	0	9154	8039
7	0	0	7574	8254
8	0	0	9535	9015
9	0	0	9907	5815

[A]Samples testing with an SP (sample-to-positive ratio) value of less than or equal to 0.299 received a 0 titer value. Titers were determined using the following equation: $\log_{10}\text{titer} = (1.72 \times \log_{10}\text{SP}) + 3.614$. Titer = antilog of $\log_{10}$ titer.

test kit, Synbiotics Co., San Diego, CA) and infectious IBDV (IBDV antibody test kit, Synbiotics Co.) were used following manufacturer's recommendations. In addition, HI tests were performed following WHO recommendations using serum samples collected at 5 and 7 dpi to detect the presence of antibodies against H5 AI.

RESULTS

Bursal lesions and seroconversion in SPF chickens infected with the IBDV E-Delaware strain. In order to establish an immunosuppressed avian animal model and to evaluate how it would affect susceptibility to AIV infection, we infected 2-day-old SPF chickens with the IBDV E-Delaware strain. IBDV was chosen instead of other immunosuppressive agents because it is endemic in most poultry producing areas of the world and represents an economically significant disease burden to the poultry industry. The E-Delaware strain was chosen because it causes immunosuppression and subclinical infection in young chickens (12). Infection of chickens with 100 CID_{50} IBDV E-Delaware strain resulted in no morbidity or mortality. Seroconversion against IBDV determined by ELISA (Table 1) along with the gross morphologic changes and severe lymphoid depletion at the microscopic level in the bursa of Fabricius confirmed IBDV infection in chickens (Fig. 1). Histologic analysis confirmed lymphoid depletion and loss of structure of the follicles in the bursa of Fabricius (Fig. 1D). A dramatic reduction in size of the bursa and the index bursa/body weight indicated atrophy of this organ (Fig. 1E). These results indicate morphologic changes in the bursa of chickens consistent with potentially compromised humoral responses. Chickens infected with IBDV that later recovered from the infection are referred throughout the text as IBDV chickens.

Table 2. Replication of mal/H5N2 (WT) virus in 3-wk-old white leghorn chickens. Results from six independent experiments in groups of three chickens inoculated by the ocular, intranasal, intratracheal, and intracloacal routes with a virus dose of 5×10^6 EID_{50}/ml.

		No. positive animals for AIV			AIV serum antibodies 14 dpi	
Experiment	Infection	3 dpi	5 dpi	7 dpi	HI	ELISA
1	LPAIV	0/3	0/3	0/3	BLD	BLD
2	LPAIV	0/3	0/3	0/3	BLD	BLD
3	LPAIV	0/3	0/3	0/3	BLD	BLD
4	LPAIV	0/3	0/3	0/3	BLD	BLD
5	IBDV + LPAIV	2/3	0/3	0/3	BLD	BLD
6	IBDV + LPAIV	1/3	0/3	0/3	BLD	BLD

[A]BLD = below level of detection.

Table 3. Virus titers in trachea and pooled lung homogenate in chickens at 3 dpi during adaptation of the mallard H5N2 virus in immunocompetent and IBDV chickens.

	Shedding in trachea			Log_{10} EID_{50} pooled lung homogenate
Passage no.	Chicken 1	Chicken 2	Chicken 3	
IBDV chickens				
P1	+	+	+	4.5
P2	+	−	−	4.5
P3	−	+	−	4.5
P4	+	+	−	5.7
P5	+	+	+	5.5
P6	+	+	+	6.5
P7	−	+	+	5.5
P8	+	+	+	6.5
P9	+	+	−	5.5
P10	−	+	+	6.0
P11	+	+	+	6.2
P12	+	+	+	6.5
P13	+	+	+	6.2
P14	+	+	+	6.2
P15	+	+	+	6.2
P16	−	+	+	6.2
P17	+	+	+	6.2
P18	+	+	+	6.7
P19	+	+	+	6.6
P20	+	+	+	6.2
P21	+	+	+	6.2
P22	+	+	+	6.6
Immunocompetent chickens[A]				
P1	+	+	+	3.5
P2	−	+	−	4.2
P3	−	−	−	BLD[B]

[A]Three independent attempts consisting of three passages. Titers represent results from the third attempt.
[B]BLD = below level of detection.

Improved replication of mal/H5N2 LPAIV in IBDV chickens. Our laboratory has been focused on understanding the molecular features that allow AIV to cross the species barrier. In this regard, we were interested in determining the replication in chickens of mal/H5N2, a mallard virus that closely resembles the H5N2 virus that caused the large HPAI outbreak in Pennsylvania in 1983 (3,8,11,28). As such, the virus represents a good model to study the events that might lead to the emergence of HPAIVs. Interestingly, despite four independent attempts using three immunocompetent, 3-wk-old chickens each time, inoculation with a dose of 5×10^6 EID_{50} of mal/H5N2 (WT) AIV resulted in neither detectable virus replication nor seroconversion, suggesting that the virus was unable to infect (Table 2; Fig. 2). In contrast, 3-wk-old IBDV chickens showed replication of the mal/H5N2 (WT) AIV after challenge (Table 2). The limited replication of the mal/H5N2 (WT) AIV suggested that IBDV infections might render chickens more susceptible to AIV infection from the wild bird reservoir. However, efficient replication of AIV, either in IBDV chickens or immunocompetent chickens, would require adaptive molecular changes in the influenza virus. Increasing the infective virus dose to 1×10^8 EID_{50} of mal/H5N2 (WT) AIV resulted in limited replication in immunocompetent chickens and increased

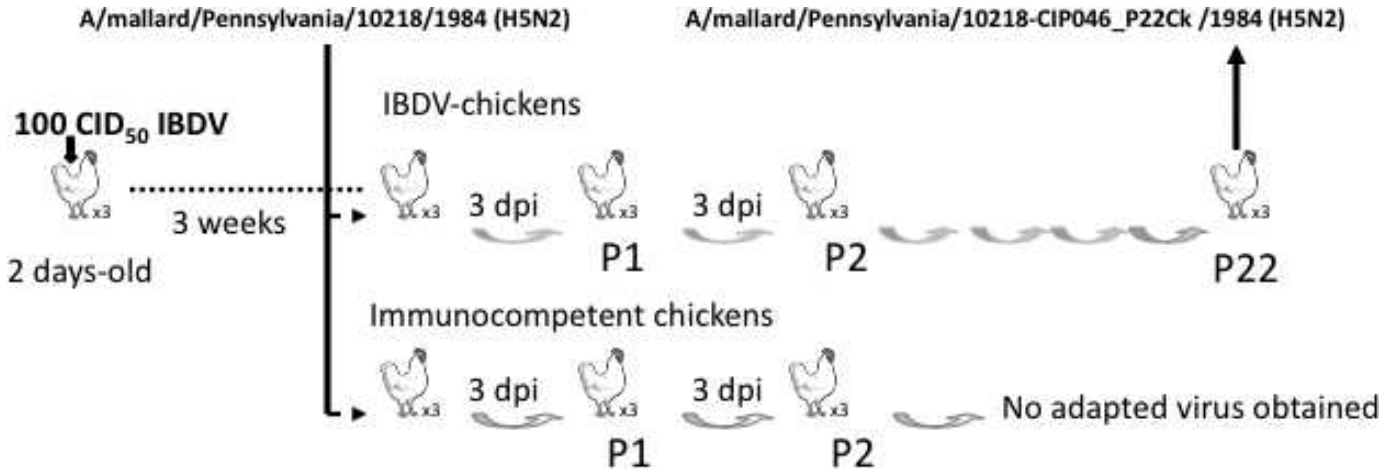


Fig. 3. Schematic representation of H5N2 LPAIV adaptation in chickens. Serial lung homogenate passage was conducted in groups of three 3-wk-old SPF immunocompetent and IBDV chickens. Only the adaptation scheme in IBDV chickens resulted in an H5N2 AIV better adapted for chickens (P22).

replication in IBDV chickens (not shown). Thus, we attempted to adapt the virus in immunocompetent chickens starting with a dose of 1×10^8 EID_{50} followed by serial lung passage. Despite three independent attempts in immunocompetent chickens, the virus was not detected in the lungs, usually by the second or third passage (Table 3). On the other hand, infection of IBDV chickens with an initial dose of 5×10^6 EID_{50} of mal/H5N2 (WT) AIV was sufficient to start an adaptation scheme that allowed serial passage of the virus contained in lung homogenates (Fig. 3). Consistent virus isolation was obtained from each lung passage of IBDV chickens infected with virus contained in the lung homogenates of the previous passage (Table 3). An increase in virus titers in lung and virus shedding in trachea was consistent during adaptation (Fig. 4 and data not shown). Eighteen passages in IBDV chickens were needed for the virus to induce consistent clinical signs. We arbitrarily terminated the adaptation scheme at passage 22 (P22) at which stage the virus not only replicated to higher titers than the mal/H5N2 (WT) AIV but also showed increased virulence in IBDV chickens. Respiratory distress, sneezing and cloudy eyes were evident in IBDV chickens inoculated with the P22-adapted AIV. Gross lesions found at necropsy corresponded with the clinical signs observed in infected chickens. Constant findings were fibrinous airsacculitis, congestion, and edema in the lungs with evident areas of pneumonia (Fig. 5).

Interestingly, both the mal/H5N2 (WT) and P22 AIVs caused lesions in the respiratory tract (lung, trachea, sinus) of IBDV chickens, although the P22 AIV showed substantially more exacerbated lesions compared to the mal/H5N2 (WT) AIV as demonstrated by the histologic analysis (Fig. 6). Lesions found in the lungs of infected birds confirmed that the P22 AIV caused severe pathology in IBDV chickens (Fig. 6). The P22 AIV caused respiratory tract lesions in 100% of the chickens. P22 AIV-induced lesions included severe diffuse bronchopneumonia characterized by fibrinoheterophilic inflammation of the parabronchus with extension into the adjacent parenchyma and severe edema. Loss of cilia and mild lymphoplasmatic inflammation were observed in the trachea and sinus. In contrast, chickens infected with the mal/H5N2 (WT) AIV showed only mild to minimal lesions in lung and other tissues were unaffected (Fig. 3). The P22 AIV also showed expanded tissue damage with lesions in the kidney, pancreas, liver, heart, conjunctiva, and thymus, which were either absent or less prominent in mal/H5N2 (WT) AIV infections (Fig. 7). Tubulointerstitial nephritis was a frequent histopathologic change observed in IBDV chickens infected with the P22 AIV (not shown). Thus, the lung passage scheme allowed for the selection of a virus with enhanced virulence. These results highlight the potential of IBDV infection in chickens to act as a facilitator in the emergence of AIVs with the ability to cause systemic spread in chickens.

The P22 AIV shows improved replication and increased virulence in immunocompetent chickens. As an additional indication that the P22 AIV was indeed more virulent than the mal/H5N2 (WT) AIV, we infected immunocompetent chickens with a dose of 5×10^6 EID_{50} of the P22 AI-adapted virus. The P22 AIV replicated efficiently in the lungs and trachea of immunocompetent chickens, in contrast to our initial observations with the mal/H5N2 (WT) AIV (Fig. 2). Remarkably the P22 AIV yielded an average of approximately 6 $\log_{10}$ EID_{50} of virus, a ~1 millionfold

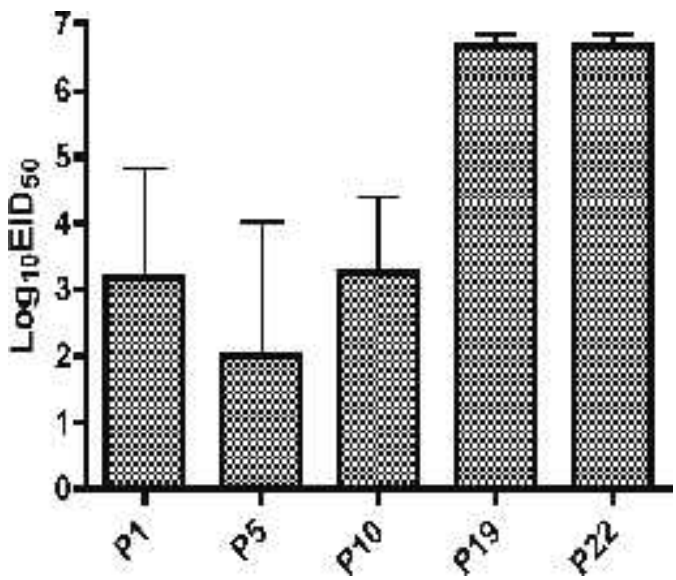
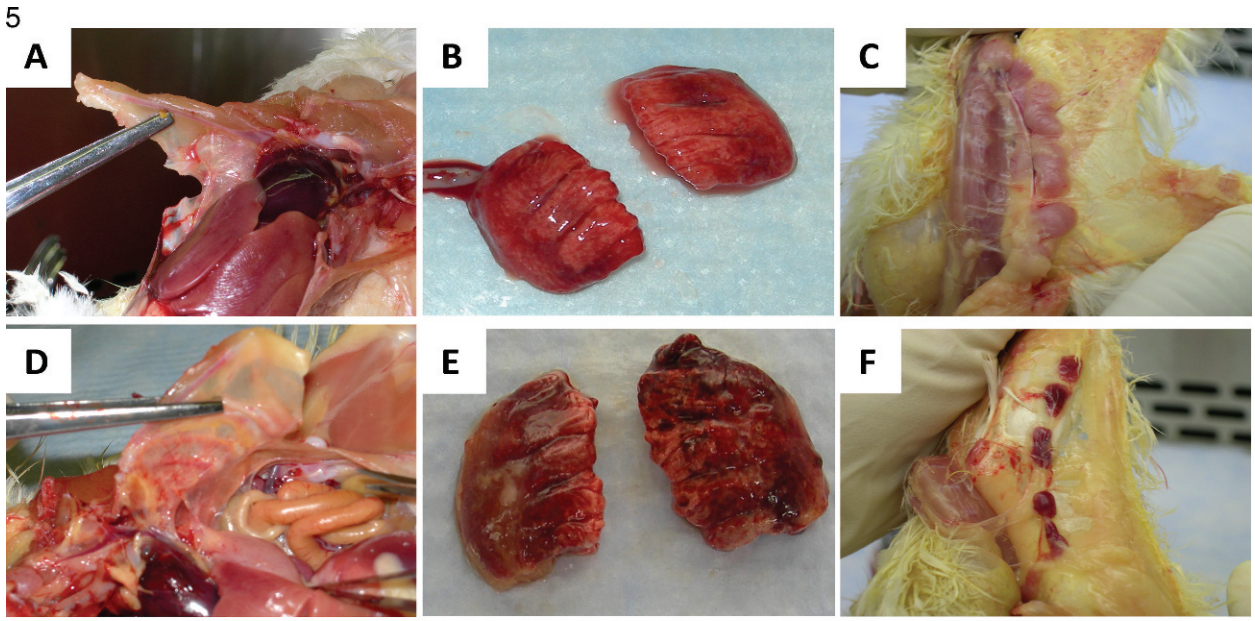


Fig. 4. Lung virus titers during adaptation of a mallard H5N2 AIV in IBDV chickens. Groups of three IBDV chickens were inoculated with a 1:10 dilution of pooled lung homogenate from the previous passage. Values represent the mean and standard deviation of virus titers in lung homogenates from three chickens per passage. Results are shown for passages (P) 1, 5, 10, 19, and 22.



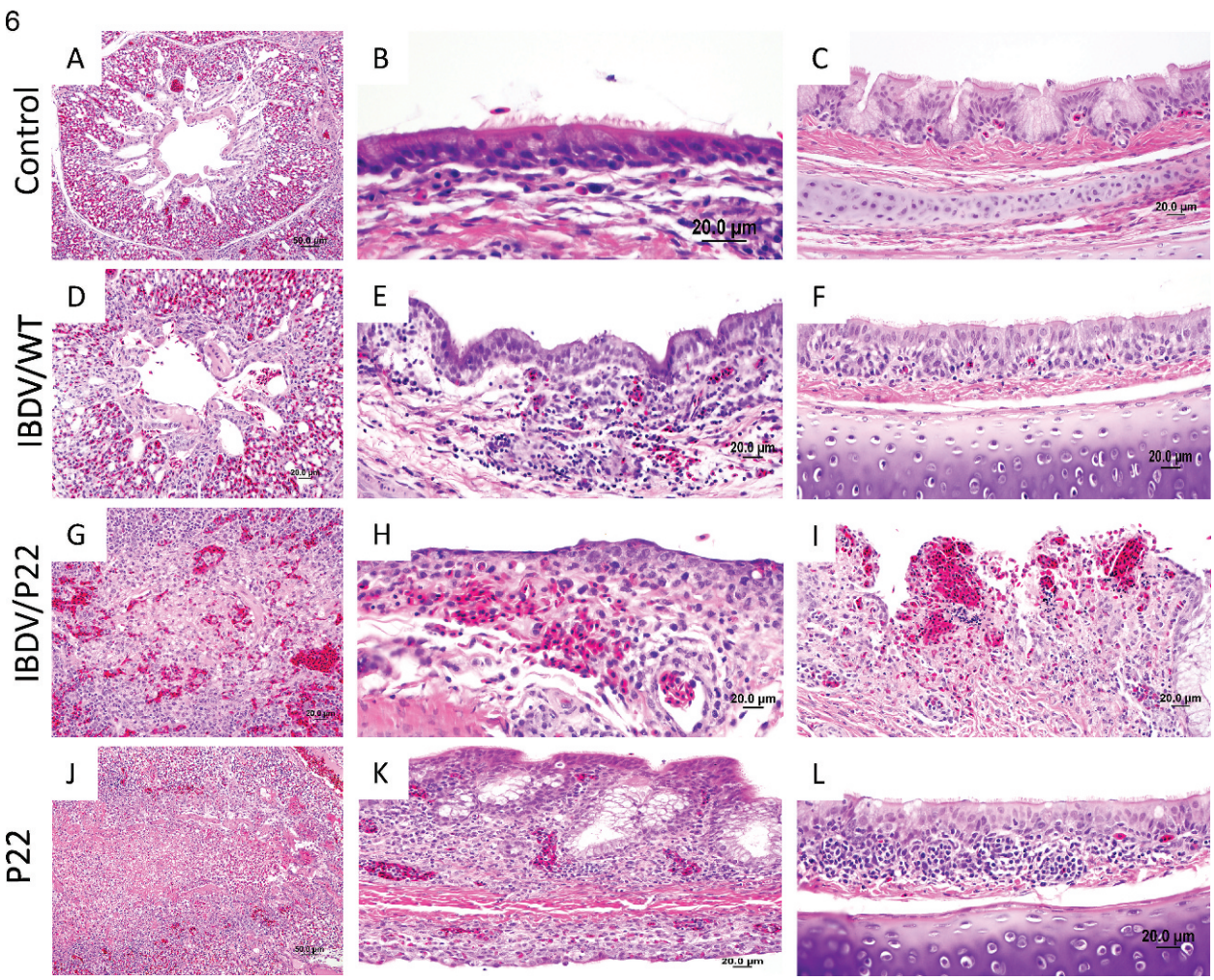


Fig. 5. Macroscopic findings found at necropsy. (A, B, and C) IBDV chickens compared to (D, E, F) IBDV chickens infected with P22 AIV. (A) The air sac is normal in 3-wk-old IBDV chickens and shows airsacculitis in 3-wk-old IBDV chickens infected with P22 AIV. (B) Lungs and (C) thymus are normal in IBDV chickens, but show (E) pneumonic lesions and (F) hemorrhages in thymus in IBDV chickens infected with P22 AIV.

Fig. 6. Histopathologic changes in the respiratory tract of chickens infected with AIV. Tissue sections from (A, D, G, and J) lungs, (B, E, H, and K) sinus, and (C, F, and L; I corresponds to larynx) trachea, were stained by H&E and analyzed for the presence of lesions. (A, B, and C) Correspond to normal tissue in 3-wk-old IBDV chickens. (D, E, and F) Correspond to IBDV chickens infected with 1×10^8 EID_{50}/ml of mal/

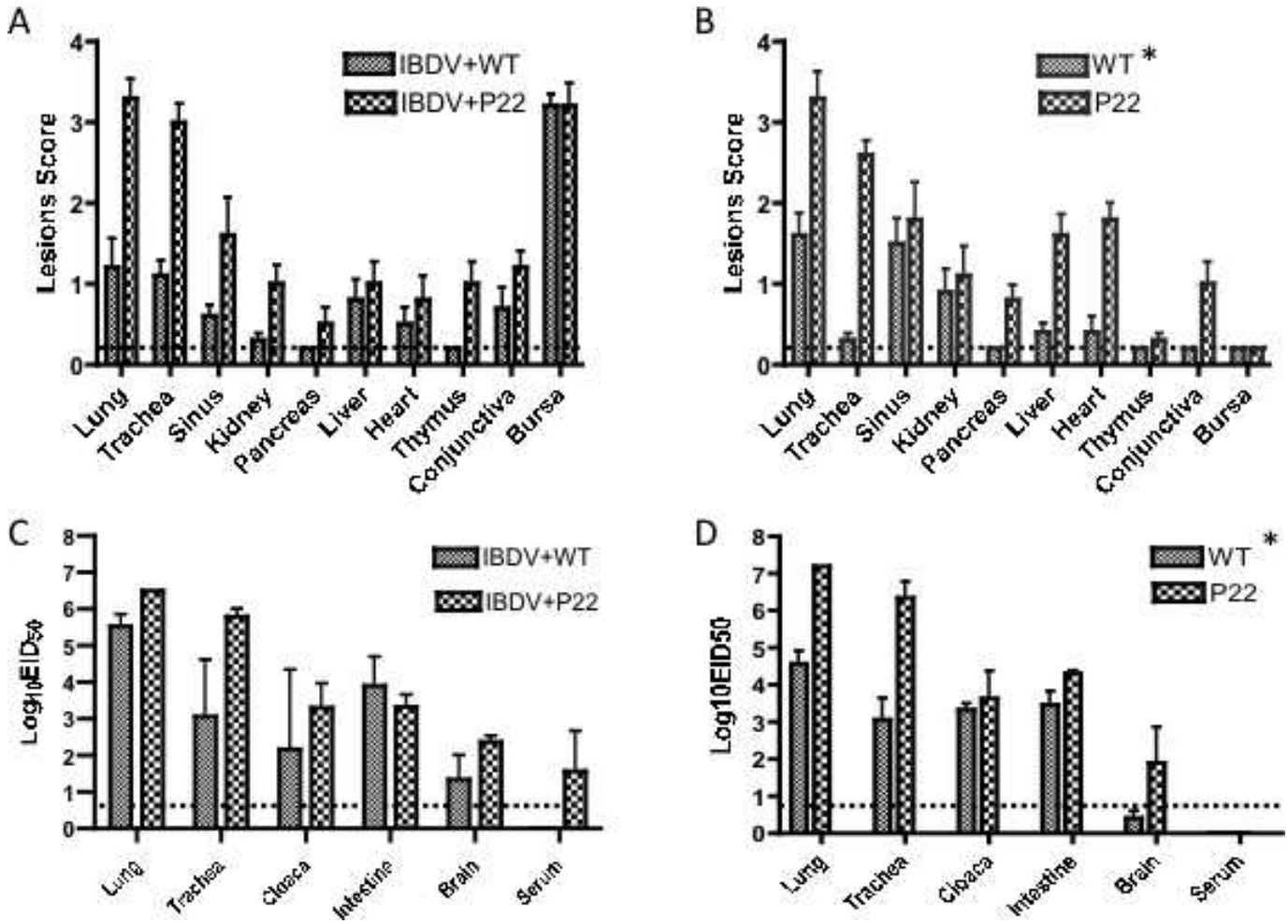


Fig. 7. Score of histologic lesions and virus titers in AIV-infected chickens. (A) Two groups of nine IBDV chickens were inoculated with 5×10^6 EID_{50}/ml of either the mal/H5N2 WT or P22 AIV. Tissue samples collected from three birds per group at 3, 5, and 7 dpi. Value of 1 to 4 were assigned according to severity of lesions observed as follows: 4: severe; 3: moderate; 2: mild; 1: minimal; 0: no lesions. Each bar represents the average of lesions score per group of nine chickens. (B) Same as in (A), but using immunocompetent chickens. *Please note that the dose of mal/H5N2 WT virus was increased to 1×10^8 EID_{50}/ml. (C) Tissue samples collected from (A) were evaluated for the presence of virus in the organs indicated. (D) Tissue samples collected from (B) were evaluated for the presence of virus in the organs indicated.

improvement compared to the WT AIV in immunocompetent chickens. Efficient replication and lesions induced by the P22 AIV infection were observed also in organs other than the respiratory tract of immunocompetent chickens (Figs. 6, 7), showing that adaptation of the H5N2 mallard AI strain in IBDV chickens resulted in a strain better adapted also for replication and increased virulence in immunocompetent chickens. Notably, the P22 virus did not induce lesions in the bursa of immunocompetent chickens but it was consistently isolated from the brain of immunocompetent chickens at slightly lower levels than in IBDV chickens. It is important to note that LPAIVs are unlikely to replicate in the brain of chickens. In this regard, the P22 AIV shows a rather unique phenotype. More importantly, the selection of an AIV strain with brain tropism may have been facilitated by prior infection with IBDV, because the mal/H5N2 WT virus itself was isolated occasionally from the brains of IBDV chickens. It remains to be determined whether the histopathologic lesions are associated with virus replication in the respective organs or if they are the result of host responses during virus infection. Such analysis is beyond the scope of the present report.

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H5N2 WT virus. Minimal inflammation in the parabronchus and mild focal loss of cilia in the sinus and mild inflammation of the sinus and tracheal mucosa was observed. (G, H, and I) Correspond to IBDV chickens infected with 5×10^6 EID_{50}/ml of P22 AIV. Severe bronchopneumonia with fibrinoheterophilic and lymphoplasmacytic inflammation in the parabronchus with congestion of capillaries and occasional hemorrhages was observed in the lungs. Sinus showed loss of cilia, flattening of epithelium, and hemorrhagic inflammation whereas the trachea showed focal necrohemorrhagic inflammation of the mucosa. (J, K, and L) Correspond to immunocompetent chickens infected with 5×10^6 EID_{50}/ml of P22 AIV. Histologic lesions were less severe than in IBDV chickens infected with the P22 AIV. Severe bronchopneumonia characterized by fibrinoheterophilic inflammation of the parabronchus with extension in to the adjacent parenchyma was observed. Sinus has loss of cilia, flattening of epithelium, and mild lymphoplasmacytic inflammation. Trachea showed occasional loss of cilia and mild inflammation in the lamina propria.

DISCUSSION

Although in recent years much attention has been focused on the study of HPAIVs, the biologic and epidemiologic significance of LPAIVs cannot be overemphasized. These viruses not only have had and will continue to have major economic impact with regard to potential losses in the poultry industry (15,23) but also as progenitors of novel strains with pandemic potential (9). Although major advances have been made, understanding of the factors that lead to the expansion or switch in host range of LPAIVs is still very limited and needs further investigation (21,26).

In this study, we questioned whether a condition that results in immunosuppression in chickens would favor improved replication of a mallard H5N2 AIV that has not been previously adapted to poultry. We attempted to mimic in the laboratory a condition that is likely to occur often in nature, IBDV infection, with resulting immune competence effects and susceptibility to infections with other agents. Comparison of the replication efficiency of the mal/H5N2 (WT) and P22 AIVs in immunocompetent and IBDV chickens indicates that the P22 AIV is better adapted for chickens than the WT mal/H5N2 AIV. The P22 AIV showed increased virulence in immunocompetent and IBDV chickens. The P22 H5N2 AIV investigated in this study showed preferential replication in the respiratory tract; this is consistent with data on influenza virus infection in mammals and birds (5,13,14, 15,22,24). It must be noted that the caveat of this study is that we performed serial passage of a virus present in lung homogenates and thus the selection process may have been biased for a virus with lung tissue tropism. Nevertheless, the results of this study clearly indicate that prior exposure to IBDV may increase the chances of AIV infection in chickens. More importantly, our adaptation scheme resulted in a strain with expanded tissue tropism even in immunocompetent chickens. Thus, it should be noted that prior infection with IBDV might allow AIV strains to infect organs or tissues that would be inaccessible under immunocompetent circumstances. Another caveat to be considered is that young broilers usually carry high levels of maternal antibodies to IBDV, thus the chances of immunosuppression may be limited. Nevertheless, the effects of IBDV on immunosuppression are not usually carefully scrutinized and should be considered as a predisposing factor to AIV infections. Such analysis is beyond the scope of the present report.

Data on virus titers from tracheal swabs and tissues (primarily lung) indicate that the adapted P22 AIV is not only more efficient for replication in IBDV chickens but also in immunocompetent chickens. The number and severity of macroscopic and microscopic lesions produced by the P22 AIV are consistent with these observations. However, we must note that the P22 AIV did not become a HPAIV strain because it did not change its pathotype in chickens according to the World Organization for Animal Health standards (not shown). Taken together, our results suggest that IBDV exposure influences the severity of lesions caused by AI, particularly if the strain is already better adapted in poultry—such as the P22 AIV strain described here. More importantly, IBDV infection early in the life of chickens—a common event in nature—allowed us to adapt an AIV in chickens in an otherwise resistant species.

In conclusion, our studies show that preexisting conditions, such as exposure to IBDV, can contribute to the mechanism of adaptation and generation of AIV strains with altered host range, tissue tropism, or virulence. Predisposing factors, such as IBDV infection, should be considered among the risk factors for the emergence of AIVs with increased pathogenic potential. This is particularly important when LPAIV strains circulate in bird populations with suboptimal immune status, which is likely to be a common event in nature.

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